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Functional and molecular characterisation of mammary side population cellsAzra J Alvi¹, Helen Clayton¹, Chirag Joshi², Tariq Enver², Alan Ashworth¹, Maria d M Vivanco¹, Trevor C Dale³ and Matthew J Smalley^{3,4}¹Breakthrough Toby Robins Breast Cancer Centre, Institute of Cancer Research, London, UK²Section of Gene Function and Regulation, Institute of Cancer Research, London, UK³Section of Cell and Molecular Biology, Institute of Cancer Research, London, UK⁴Current address: Division of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The NetherlandsCorresponding author: Trevor C Dale (e-mail: trevor@icr.ac.uk)

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Breast Cancer Res 2003, **5**:R1-R8 (DOI 10.1186/bcr547)© 2003 Alvi *et al.*, licensee BioMed Central Ltd (Print ISSN 1465-5411; Online ISSN 1465-542X). This article is published in Open Access: verbatim copying and redistribution of this article are permitted in all media for any non-commercial purpose, provided this notice is preserved along with the article's original URL.**Abstract****Background:** Breast cancer is thought to arise in mammary epithelial stem cells. However, the identity of these stem cells is unknown.**Methods:** Studies in the haematopoietic and muscle systems show that stem cells have the ability to efflux the dye Hoechst 33342. Cells with this phenotype are referred to as the side population (SP). We have adapted the techniques from the haematopoietic and muscle systems to look for a mammary epithelial SP.**Results:** Of mammary epithelial cells isolated from both the human and mouse mammary epithelia, 0.2–0.45% formed a

distinct SP. The SP was relatively undifferentiated but grew as typical differentiated epithelial clones when cultured. Transplantation of murine SP cells at limiting dilution into cleared mammary fat pads generated epithelial ductal and lobuloalveolar structures.

Conclusion: These data demonstrate the existence of an undifferentiated SP in human and murine mammary epithelium. Purified SP cells are a live single-cell population that retain the ability to differentiate *in vitro* and *in vivo*. Studies of haematopoietic cells have suggested that the SP phenotype constitutes a universal stem cell marker. This work therefore has implications for mammary stem cell biology.**Keywords:** breast epithelium, differentiation markers, side population, stem cells, telomerase**Introduction**

Breast cancer affects one in 10 women in Western countries. Current theories suggest cancer is a disease of stem cells [1], and it is therefore important to identify breast epithelial stem cells as potential targets both for therapeutic intervention and for breast cancer prevention. Transplantation studies in the mouse have shown that stem cells are present throughout mammary ductal and lobular structures, and may be particularly enriched in end buds, the rapidly growing tips of the emerging ductal network [2]. Analysis of mouse mammary tumour virus (MMTV) retroviral integration sites has shown that a complete

mammary gland can develop from the progeny of a single cell, but the identity of this cell is unclear [3]. Undifferentiated cap cells at the tips of the end buds have been suggested to be stem cells, largely based on their location [2]. Morphological studies have also suggested that undifferentiated small light cells distributed within mammary ducts may be stem cells [4]. Functional data confirming these assignments have been missing due to the lack of appropriate cellular markers that can be used to isolate live cells. Two main developmental motifs (ductal and lobular) and two main cell types (myoepithelial and luminal) are found within the mammary gland. However,

BCRP = breast cancer resistance protein; bp = base pairs; DMEM = Dulbecco's modified Eagle's medium; FACS = fluorescence-activated cell sorter; FCS = foetal calf serum; H & E, haematoxylin and eosin; MMTV = mouse mammary tumour virus; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase-polymerase chain reaction; SP = side population; TERT = telomerase catalytic subunit.

the relationship between stem cells and these cell populations remains unclear.

Flow cytometric analysis of haematopoietic and muscle cells has shown that Hoechst dye exclusion defines a side population (SP) of stem cells for both tissues [5,6]. It has been postulated that the SP is a universal stem cell phenotype [6]. We have now identified human and mouse breast epithelial SP cells and have shown that they constitute an undifferentiated subpopulation that can differentiate into ductal and lobular structures and into myoepithelial and luminal epithelial cell types.

Materials and methods

Isolation of human epithelial cells

Breast tissue derived from reduction mammoplasties was cut up manually into small pieces (approximately 0.5 cm cubed) and digested overnight at 37°C in a shaking incubator with 0.5–1 mg/ml collagenase (Type I; Sigma, Poole, UK) in phenol-red free DMEM (Gibco, Paisley, UK) supplemented with 5% charcoal-treated serum. Following enzyme digestion, the fat layer was decanted and the epithelial pellet was washed several times with DMEM medium. The breast organoids (ductal and lobuloalveolar structures) were isolated from red blood cells, fibroblasts and endothelial cells by sequential filtration and backflushing from 140 and 53 µm pore size polyester monofilament meshes (Locker Tex, Warrington, UK) as described by Hallows *et al.* [7]. The pooled organoids were disaggregated by pipetting in 0.25% trypsin-EDTA (Sigma), followed by filtration through a 40 µm mesh (Becton Dickinson, San Jose, CA, USA) to yield a predominantly single-cell suspension.

Isolation of mouse mammary epithelial cells

Primary mouse mammary epithelial cells were harvested essentially as described by Smalley *et al.* [8], with modifications described by Naylor *et al.* [9]. In brief, the fourth mammary fat pads were removed from 8-week-old to 10-week-old virgin female FVB mice and were subjected to mechanical and enzymatic digestion to obtain epithelial 'organoids'. Contaminating fibroblasts were removed by differential plating and the organoids were enzymatically digested to single cells. Primary cells were then directly processed for Hoechst staining without intervening culture.

Isolation of mouse bone marrow cells

Femurs from FVB mice were dissected out. The ends of each bone were snipped off with dissecting scissors, and a 5 ml syringe containing PBS and equipped with a 25 G needle (Terumo, Leuven, Belgium) was used to flush out the bone marrow from both ends of the bone. The resulting diluted bone marrow was washed with L15/10% FCS and pelleted. Red cells were lysed by resuspending the pellet in 1 ml red blood cell lysing buffer (Sigma), pipetting

up and down briefly, and incubating for 5 min at 37°C. If red cells were still present after washing (red colour in the pellet), the lysis procedure was repeated once more.

Clonal culture of primary mouse mammary epithelial cells

For analysis of the morphology and the cloning efficiency, mouse mammary epithelial cells were plated at 2000 cells per flask in 25 cm² tissue culture flasks containing 5×10^5 irradiated (20 Gy) 3T3-L1 mouse fibroblast (ATCC, Bethesda, MD, USA) feeders. Cultures were maintained in a 1:1 mix of DMEM and Ham's F12 (Gibco) supplemented with 10% FCS, 5 µg/ml insulin, 10 ng/ml cholera toxin (Sigma) and 10 ng/ml epidermal growth factor (Sigma). Cultures were kept in a 90% nitrogen/5% carbon dioxide/5% oxygen environment for optimal clonal growth as previously described [8]. After 8 days in culture, flasks were fixed in 0.5% glutaraldehyde in PBS, clones were photographed and the flasks were then stained with haematoxylin to allow counting of colony numbers. To assess levels of contaminating fibroblasts in the cell preparations, cells were plated at clonal density in 25 cm² flasks with 10T1/2 (ATCC) feeders that expressed β-galactosidase (to distinguish them from any contaminating fibroblasts), or at 4×10^4 per 25 cm² flask without feeders, in DMEM/10% FCS, and maintained in 5% CO₂/air only. To assess levels of contaminating haematopoietic cells, mouse mammary SP cells were cultured under conditions previously established for the culture of bone marrow-derived cells [10].

Antibodies and reagents

All reagents came from Sigma unless otherwise stated. Antibodies LL002 (anticytokeratin 14), LE61 (anticytokeratin 18) and LP2K (anticytokeratin 19) were kind gifts from EB Lane. Antibodies 33A10 (anti-epithelial membrane antigen) and GoH3 (anti-α6 integrin) were kind gifts from A Sonnenberg. Antivimentin V9 and anti-α-isoform smooth muscle actin 1A4 were purchased from Sigma. Anti-CD45 antibody I3/2.3 and fluorochrome-conjugated secondary antibodies were purchased from Cambridge Bioscience (Cambridge, UK). The antitelomerase catalytic subunit antibody was purchased from Calbiochem (Nottingham, UK). The anti-oestrogen receptor antibody was purchased from DAKO (Ely, UK).

Hoechst 33342 staining of cells

Mouse bone marrow and epithelial cells were resuspended at 10⁶/ml in L15/10% FCS prewarmed to 37°C (DMEM/10% FCS for human breast epithelial cells), and Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) was added at a final concentration of 5 µg/ml. Verapamil (Fluka; Sigma) was added to samples at a final concentration of 20 µM, and was more toxic to mouse mammary SP cells than to bone marrow cells or human mammary SP cells (data not shown). Cells were incubated

for 90 min at 37°C with occasional agitation. After incubation, cells were washed with cold medium and resuspended at $(2-3) \times 10^6/\text{ml}$, and propidium iodide (Molecular Probes) was added to a final concentration of 2 $\mu\text{g}/\text{ml}$.

Flow cytometry

Cells were sorted on a BD FACSVantageSE cell sorter (Becton Dickinson) either into tubes, onto poly-L-lysine-coated slides (BDH; Merck Ltd, Lutterworth, UK), or into culture plates using the CloneCyto system. The machine is equipped with two Coherent 90 C-4 argon ion lasers (Coherent, Santa Clara, CA, USA), one with visible optics set to 488 nm and one with multiline UV optics (333.6–333.8 nm). Hoechst 33342 fluorescence was measured at both 424/44 nm and above 670 nm (split by a 610 nm short-pass dichroic mirror), both from UV excitation. Dead cells were excluded by propidium iodide fluorescence measured at 564–606 nm.

Immunofluorescence analysis

Cells were plated at a density of 500/well on glass coverslips set up in 24-well plates with 2000 irradiated feeders per well, using the same growth conditions as already described. After 8 days, coverslips were fixed in ice-cold 50:50 methanol:acetone for 5 min, and then stained. Alternatively, cells were sorted directly onto poly-L-lysine-coated slides, and then air-dried and fixed as already described. Cells were stained by multiple indirect immunofluorescence as described elsewhere [8] using mouse and rat primary monoclonal antibodies against the antigens presented in Table 1, and species, class and subclass-specific secondary antibodies conjugated to fluorescein isothiocyanate or Texas Red (Cambridge Bioscience). No first antibody controls were used to screen for nonspecific binding of secondary antibodies. Additional controls involved showing that several primary antibodies were negative for staining to mouse mammary SP cells. These included anti-Ep-CAM (Ab4 Clone ESA 43; IgG₁), anti-CD34 (RAM34; IgG_{2a}K), anti-c-Kit (CD117, clone 2b; IgG_{2b}K) and FLK1 (IgG_{2a}). Stained cultures were observed using a Zeiss Axiovert inverted microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) and were photographed. Cell counts taken from the photographs were used to determine the percentage of brightly stained cells.

Cleared fat-pad transplantation

Transplantation into epithelial-free fourth mammary fat pads of 21-day-old FVB mice was carried out as described previously [9,11,12]. Freshly harvested SP and non-SP cells were transplanted at varying numbers as already described. Transplants were given 8 weeks to grow before either being harvested or allowing host animals to be mated. Transplanted fat pads from mated animals were harvested on days 17–18 of pregnancy. Harvested glands were fixed and carmine-stained as

Table 1

Side population (SP) proportion and hormonal status

Age (years)	SP (%)	Term pregnancy	Contraception	Day of menstrual cycle
33	0.03	None	COC	8/28
43	0.68	–	–	–
39	0.47	4 (breast fed)	None	21/28
40	0.07	None	None	15/28
19	0.14	None	COC	14/28
22	0.15	None	DeproProvera	N/A
39	0.07	1 (breast fed)	None	20/–*
43	0.01	2 (breast fed)	None	14/28
57	0.03	2 (breast fed)	None	8/21

Relating percentage of SP found in nine different patients with age, number of pregnancies, use and type of contraceptives and day of menstrual cycle. COC, combined oral contraceptive. * Irregular cycle length.

whole mounts. After being photographed, pieces of interest were dissected out of the whole mounts, embedded in paraffin blocks and processed by standard histological techniques for H & E sections and immunocytochemistry.

RT-PCR analysis of ABC transporter proteins

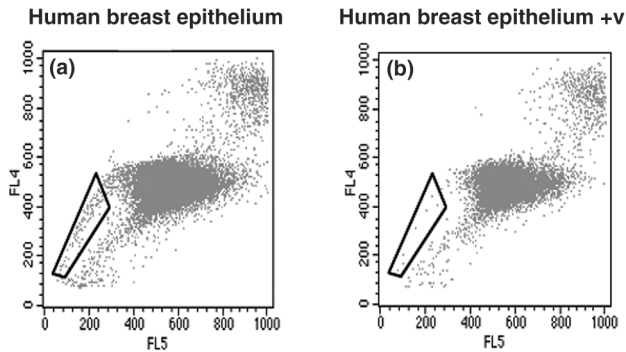
For RT-PCR analysis of multidrug-resistant protein expression in mammary epithelial cells, RNA was isolated using Rneasy spin columns (QIAGEN Ltd, Crawley, UK). RT reactions were carried out on RNA from approximately 1000 cells using Sensiscript RT (QIAGEN) and an oligo dT primer according to the manufacturer's instructions. Control reactions with no enzyme were carried out in parallel. PCR amplification was carried out on one-fifth of the RT or control reactions. The expression of four ABC transporter proteins (breast cancer resistance protein 1, multidrug resistance-associated protein 1, multidrug resistance-associated protein 3 and multidrug resistance-associated protein 4) was analysed; primer sequences for these were kindly provided by Brian Sorrentino [6].

Results

Identification of human and murine SP cells

Epithelial cells isolated from nine independent, normal human breast samples were stained with Hoechst and analysed by FACS. Each preparation contained a small fraction of cells ($0.18 \pm 0.23\%$) that exhibited a SP (Fig. 1a). The formation of the SP population was blocked by 20 μM verapamil, consistent with previous studies showing a requirement for ABC transporter family function in the SP phenotype (Fig. 1b) [5]. The SP was found in all samples regardless of age, parity, contraceptive status or day of menstrual cycle (Table 1).

Figure 1



Flow-cytometric analysis of human breast epithelial cells stained with 5 μ M Hoechst 33342. (a) Trace demonstrating the presence of a side population (box). (b) Addition of 20 μ M verapamil (+v), resulting in a 12-fold reduction in the side population. X axis, Hoechst red fluorescence intensity (FL5); Y axis, Hoechst blue fluorescence intensity (FL4).

We next examined whether the SP was present in mouse mammary epithelial cells. Results from 17 independent experiments showed that $0.45 \pm 0.22\%$ of mouse mammary epithelial cells had the SP phenotype (Fig. 2a). This proportion of cells exhibiting the mammary SP phenotype was similar to that observed in similarly stained mouse bone marrow samples ($0.14 \pm 0.11\%$) (Fig. 2b) and was in line with previous studies [5]. Representative flow cytometry traces of Hoechst-stained cells from mouse mammary epithelium and mouse bone marrow are shown in Figure 2a,b. The increased availability of SP cells

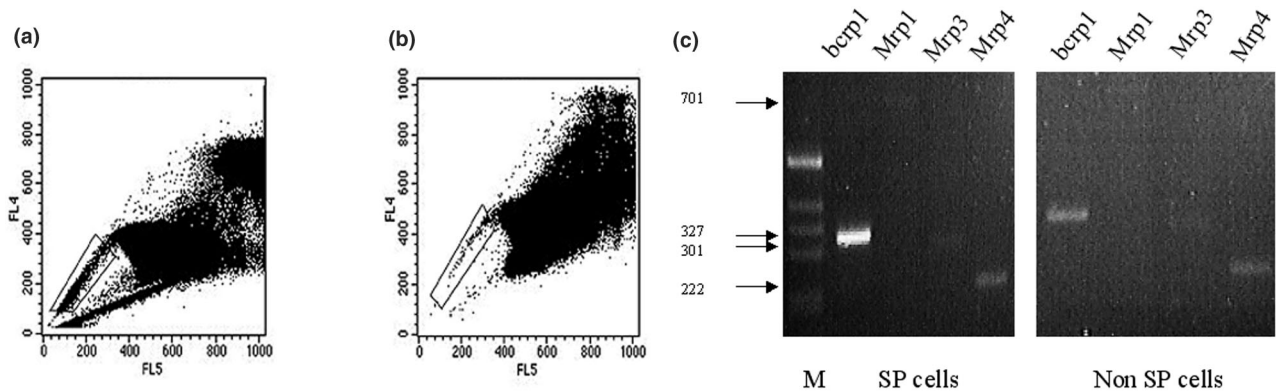
from the mouse mammary epithelium encouraged us to move to a mouse model for subsequent studies.

Markers of SP mammary epithelial cells

The SP phenotype is thought to arise through the action of ABC transporter cassette proteins, and in particular ABCG2/breast cancer resistance protein 1 [6]. RT-PCR analysis of mouse mammary SP cells confirmed the expression of breast cancer resistance protein 1, as well as three other members of the ABC transporter family (multidrug resistance-associated protein 1, multidrug resistance-associated protein 3 and multidrug resistance-associated protein 4) at lower levels (Fig. 2c).

To immunophenotype mouse mammary epithelial SP and non-SP cells, they were directly sorted on to poly-L-lysine-coated slides and stained by indirect immunofluorescence (Table 2). The results suggest that SP cells are a relatively undifferentiated population that express lower levels of cytokeratins and higher levels of vimentin than non-SP cells. Vimentin expression is not exclusive to fibroblasts and has previously been described in mammary epithelial cells [13]. Levels of CD45-expressing cells were low in the SP fraction while CD34 and Flk1 were not expressed, suggesting that mammary SP cells were not significantly contaminated with blood stem cells. Similar proportions of cells expressed the oestrogen receptor in both SP and non-SP cells. Interestingly, the SP fraction was enriched for cells that express the catalytic subunit of telomerase [14]. The results from these studies, while not yet statistically significant, have important mechanistic implications for the origin of oestrogen receptor-positive and oestrogen receptor-negative tumours, and require further investigation.

Figure 2



Analysis of mouse side population (SP) cells. Flow cytometric analysis of mouse cells stained with Hoechst 33342: (a) SP in mouse mammary epithelial cells (box), (b) SP in mouse bone marrow cells. X axis, Hoechst red fluorescence intensity (FL5); Y axis, Hoechst blue fluorescence intensity (FL4). (c) RT-PCR analysis of ABC transporter cassette proteins in SP and non-SP cells from mouse mammary epithelial cells. M, molecular weight marker; Brcp1, breast cancer resistance protein; Mrp, multidrug resistance-associated protein. Sizes of bands: Brcp1, 327 bp; Mrp1, 701 bp; Mrp3, 301 bp; Mrp4, 222 bp.

Table 2**Antibody staining profiles**

	Cytokeratin 14 (clone LL002)	Cytokeratin 19 (clone LP2K)	Vimentin	Epithelial membrane antigen (clone 33A10)	α 6-Integrin	Oestrogen receptor (clone 1D5)	CD45	Telomerase (clone α -TERT)
SP	0.5 (567)	5 (309)	63 (194)	4 (356)	44 (396)	41 (108)	5 (290)	35 (99)
Non-SP	15 (265)	39 (144)	23 (100)	16 (102)	10 (181)	56 (113)	40 (211)	5 (86)

Data presented as the percentage of bright cells (number of cells counted). Summary of staining profiles for mouse mammary epithelial side population (SP) and non-SP cells.

***In vitro* and *in vivo* differentiation of SP cells**

To characterise the *in vitro* differentiative potential of the mammary SP, cells were plated under conditions designed to promote the growth of mammary epithelial cells, haematopoietic colonies or fibroblasts. Haematopoietic culture conditions supported growth of bone marrow-derived SP cells, but they did not support the growth of mammary-derived SP cells (data not shown). Similarly, fibroblast culture conditions supported the growth of primary mouse fibroblasts, but there was no growth of fibroblast-type cells under such conditions in cultures in which either SP or non-SP cells had been plated, either at clonal density or in bulk culture (data not shown). It thus seems unlikely that contaminating haematopoietic cells or fibroblasts make up the bulk of the SP fraction seen in mouse mammary epithelial cell preparations. By contrast, culture of both SP and non-SP cells under conditions previously optimised for mouse mammary clonal culture resulted in the growth of mouse epithelial clones [8], with mean cloning efficiencies of 4.7 ± 0.55 and $2.1 \pm 1.6\%$, respectively (2000 cells plated per flask; $n = 5$).

The morphology and ratios of the clone types were consistent with the type A–D classification found when unsorted primary mammary epithelial cells were cloned [8,15]. Identical clone types and ratios of types A–D were observed following growth of SP and non-SP preparations.

Double immunofluorescence staining of clones, using the antibodies LE61 and LP2K (anticytokeratin 18 and anticytokeratin 19, respectively; *in vivo* markers of mammary luminal epithelial cells) and LLO02 (anticytokeratin 14; a marker of mammary myoepithelial cells) [8,15], demonstrated that SP-derived and non-SP-derived clones had identical staining patterns. All were uniformly strongly positive for cytokeratin 18, and most cells within clones also double stained for cytokeratin 14. Staining for cytokeratin 19 was more heterogeneous. Occasional cells, apparently lying below the clonal ‘monolayer’, were cytokeratin 14-positive only (data not shown). Such promiscuous patterns of cytokeratin expression in mammary epithelial-derived clones are fully consistent with our previous data and suggest that SP cells differentiate into classic mammary epithelial cell clones following *in vitro* culture.

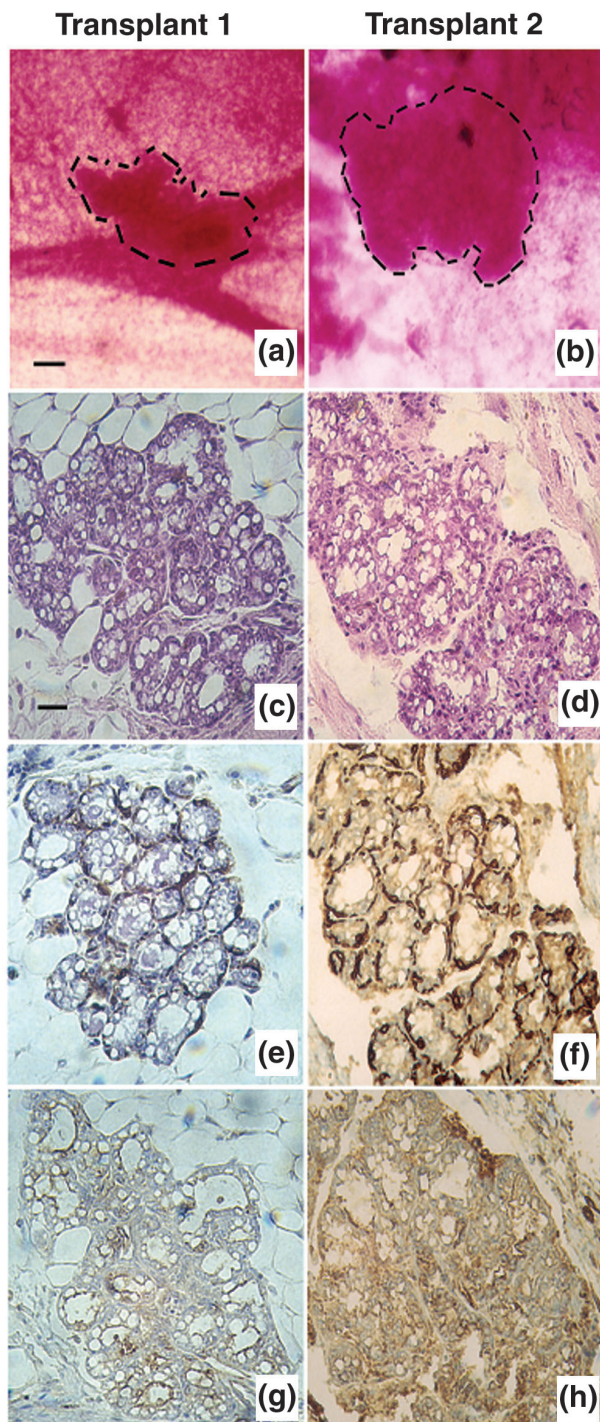
To examine their *in vivo* differentiative potential, SP cells that had been freshly isolated and had not undergone any intervening culture period were transplanted at $<5 \times 10^3$ cells per fat pad into mammary fat pads of syngeneic animals from which the endogenous epithelium had been removed (‘cleared’) [11]. Transplants were given 5–8 weeks to develop and then animals were mated. Transplanted glands were harvested on days 17–18 of pregnancy.

Outgrowths were observed in five of 37 SP transplanted fat pads. These outgrowths consisted of four lobuloalveolar structures and one ductal–lobular outgrowth (ducts and lobules) according to the classification of mammary transplant outgrowth types of Kordon and Smith [3]. Six out of 25 non-SP transplanted fat pads contained outgrowths, although comparisons between SP and non-SP transplant rates may be unwarranted (see Discussion). H & E staining of sections through the SP outgrowth lobuloalveolar structures (Fig. 3a–h) showed that they consisted of a number of tightly packed alveoli with central lumina surrounded by layers of luminal epithelial and myoepithelial cells. Staining for markers of myoepithelial and luminal epithelial cells (α -smooth muscle actin and cytokeratin 19) confirmed the identity of myoepithelial and luminal epithelial cells, respectively [8,15].

Discussion

We have demonstrated the presence of SP cells within the human and mouse mammary glands. SP cells were undifferentiated, and they generated ductal and lobular structures containing both myoepithelial and luminal epithelial cell types upon transplant *in vivo*. Mammary SP cells were detected in all human breast samples studied, although no correlation was detected between age, parity, contraceptive status or day of menstrual cycle. It is possible that subtle changes in SP frequency were masked by the experimental variables inherent in preparing primary breast material for FACS analysis. The inhibition of the SP phenotype by verapamil together with the expression of breast cancer resistance protein (BCRP) suggest that the BCRP-dependent transporter function could be a major contributor to the ability of mammary SP cells to export Hoechst dye [6]. Recent studies suggest that BCRP expression in the normal human breast is heterogeneous

Figure 3



Histology and immunocytochemistry of two representative side population (SP) outgrowths. (a), (b) Wholemount morphology of carmine-stained outgrowths from transplanted mouse mammary side population cells. Lobuloalveolar structures indicated by dashed outlines. Bar = 750 μ m. (c), (d) H & E-stained section of SP outgrowth. (e), (f) Immunocytochemical staining of the section of SP outgrowth for the myoepithelial cell marker α -smooth muscle actin. (g), (h) Immunohistochemical staining of the section of SP outgrowth for the luminal epithelial cell marker cytokeratin 19. (c)–(h) Bar = 350 μ m.

[16]. However, further work will be required to determine whether BCRP expression can be used as a molecular marker for SP/stem cells. With the development of appropriate reagents, the relationship between SP/stem cell phenotype and breast cancer risk could be explored.

Freshly isolated SP cells expressed low levels of differentiated markers for both luminal (epithelial membrane antigen and cytokeratin 19) and myoepithelial cell types (cytokeratin 14). Oestrogen receptor expression was detected in SP cells, suggesting that these cells have the capacity to respond to the proliferative effects of oestrogen. Significantly, expression of the telomerase catalytic subunit was higher in SP compared with non-SP cells. The telomerase catalytic subunit mRNA is expressed within mitotically inactive regions of breast terminal ducts and lobules, and is associated with stem cell compartments in other tissues [17]. An increase in telomerase activity is one of the earliest events in breast tumourigenesis and has been shown to drive cellular immortalisation [17,18].

While the present article was under review, Welm and colleagues published a report showing that Sca-1-positive cells were enriched for stem cell precursors and that mouse mammary SP cells expressed high levels of Sca-1 [19]. Our data confirm their observation that SP cells are present in the mouse mammary gland, and we have subsequently shown that Sca-1 is differentially expressed in SP versus non-SP cell preparations (15.8% SP:1.8% non-SP in cells from FVB mice). These percentages were comparable with those observed in the B6/129 mice characterised by Welm *et al.* [19]. Unlike Welm *et al.*, however, we directly recovered sufficient live SP cells to allow an analysis of primary SP cell transplantation potential and *in vitro* growth potential.

Following *in vitro* culture, murine SP cells express classic mammary epithelial markers including cytokeratin 14, cytokeratin 18 and cytokeratin 19, suggesting that culture conditions promote differentiation. Previous studies have shown that unsorted epithelial cells grown under these conditions retain the ability to repopulate the mammary gland upon transplantation [20]. However, the identity of the clonal type(s) that retain *in vivo* stem cell potential remains unclear. When unsorted primary epithelial cells were cultured in bulk for 8 days prior to Hoechst staining, the classic SP FACS profile was not observed (data not shown). The loss of the SP phenotype may be due to a reduction in the proportion of SP cells following culture. Alternatively, the ability of cells to exclude Hoechst dye may be increased by *in vitro* culture such that the SP fraction can no longer be distinguished. Considerable cellular heterogeneity exists within three of the four previously described epithelial clone types (types A–D) [8,15], and it is probable that only some cells within the colonies retain the ability to repopulate the mammary gland.

Transplantation of SP cells at limiting dilution generated lobuloalveolar and ductal-lobular structures at low frequency, which is an encouraging finding given that as few as 2000 cells were used. Non-SP cells also generated outgrowths. In the current study, the relative rates of SP transplantation versus non-SP transplantation should not be directly compared because very low numbers of SP cells were generated in each primary cell preparation and because comparisons between batches of primary cells were hampered by the toxic effects of Hoechst staining (data not shown). The morphology of the ductal-lobular and lobuloalveolar structures derived from SP cells resembled that previously described for mammary epithelial clone types *in vivo* [3]. The lobular structures had a histology that was consistent with the late stage of pregnancy from which they were isolated, and both myoepithelial and luminal cells were expressed in the appropriate cellular layers. These data together suggest that mammary SP cells retain a full differentiative and developmental potential.

If SP cells were pure mammary stem cells, it might be expected that they would be highly enriched for the ability to repopulate the fat pad following transplantation, as has been shown for Sca-1-enriched cells [19]. However, a direct comparison with Sca-1 enrichment studies may not be valid. One of the assumptions underlying mammary transplant studies is that mammary outgrowths at limiting dilution are clonal (14,000–20,000 cells per cleared fat pad) [21]. Recent data show that this assumption may be incorrect. When a population of epithelial cells containing 10% lacZ-positive marked cells was transplanted at limiting dilution, all transplant outgrowths were found to contain mixtures of lacZ-positive and lacZ-negative cells [22]. As the rate of transplants 'takes' in these experiments was normal, this suggests that more than one cell may be required for a successful transplant [22]. In this context, the study of Sca-1-enriched cells [19] may not be comparable with that of SP cells, because SP cells comprise 0.2–0.45% of epithelial cells while Sca-1 cells are 100 times more common, comprising 20% of the total mammary epithelial population. Thus, 99.7% of non-SP cells may contain additional (Sca-1-positive) cells that enhance the rate of transplantation [21] or directly contribute to a polyclonal outgrowth. A key study showing polyclonal MMTV-infected glands become monoclonal with respect to the site of MMTV integration following serial transplantation [3] provided evidence for the presence of single totipotent mammary stem cells, but did not show that the single cell was able to generate complete outgrowths at limiting dilution.

In the longer term, approaches to identify surface markers and strategies for isolating healthy SP-like cells need to be developed to allow the clear identification of putative stem cells. In addition, a better understanding of the clonal composition of outgrowths should allow the use of

mammary transplantation as a quantitative assay for stem cell enrichment.

Conclusion

We have identified human and mouse SP cells. We show that mammary SP cells are an undifferentiated subfraction of total epithelial cell preparations. SP cells retain the potential to differentiate into typical mammary clones *in vitro* and into normal lobular and ductal structures *in vivo*. As haematopoietic SP cells are enriched for stem cell precursors, the present data is of direct relevance to the identity of mammary stem cells.

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